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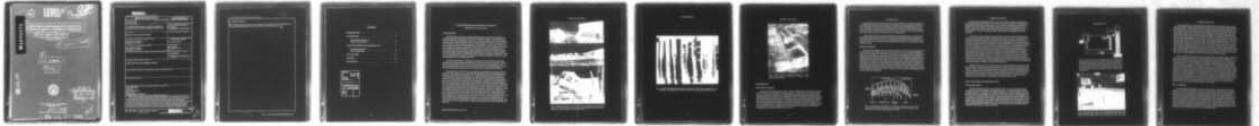
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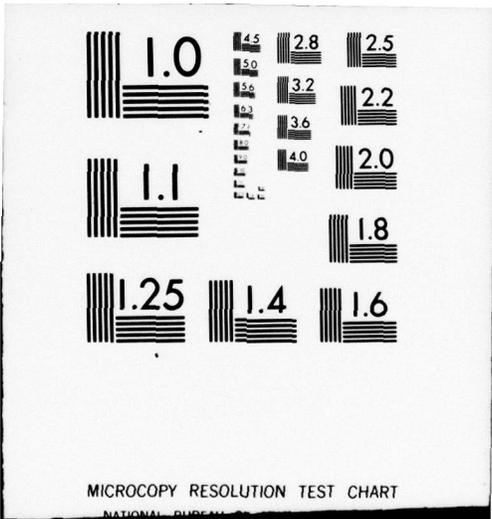
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A Closed Recirculating Aquarium System for Laboratory Culture of *Limnoria tripunctata*.

10 KATHLEEN K. PARRISH AND JOHN D. BULTMAN  
Marine Biology and Biochemistry Branch  
Ocean Sciences Division

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20. ABSTRACT (Continue on reverse side if necessary and identify by block number) The Navy is studying the nature of the antiborer constituents of naturally marine-borer-resistant woods to determine if these natural products could be developed into new, more effective, nonpolluting wood protectants against these borers; emphasis is placed on those compounds effective against limnoria. In order to study the effect of these natural wood extractives on the physiology of limnoria under controlled laboratory conditions, a laboratory culture of <i>Limnoria tripunctata</i> has been established using a closed recirculating aquarium system. The usual invertebrate closed-aquarium culture techniques have been modified to fit our needs. This includes the redesign of the biological filter bed		

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and its location outside each aquarium. A brief biology of the limnarians and a full description of our closed recirculating aquarium system and its procedures for maintenance are given.

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**A Closed Recirculating Aquarium System for Laboratory  
Culture of *Limnoria tripunctata***

**INTRODUCTION**

Besides being an important renewable natural resource, wood is more versatile and generally cheaper to use than other construction materials. Because of its desirable properties, the Navy uses large quantities of wood for various purposes: utility poles, cross-ties, buildings, piers, wharves, dolphins, camels, and fender systems. However, unless protected from a variety of wood-destroying organisms, the durability of most of the conventional construction woods is poor, particularly wood in marine service. For example, Fig. 1 shows the extreme borer damage wrought by wood destroyers in the San Francisco Bay area around 1920 [1]; Fig. 2 shows the typical hour-glass effect caused by limnoria, marine crustacean isopods, as they damaged pilings supporting a pier; and Fig. 3 shows a fender timber thoroughly infested by teredinids, marine molluscs. Although it is difficult to assess the annual expenditure for repair or replacement of borer-damaged wood, the most recent estimate places the cost at \$200 million [2].

The Navy is interested in developing new methods of wood protection which will diminish this cost and ensure an improved service life for its wooden structures. This interest has been intensified recently because creosote is inadequate as a protectant in some marine situations, and creosote and other currently used wood preservatives have an uncertain future resulting from the growing national concern for environmental pollution.

The search by the Navy for new, more effective, nonpolluting wood protectants has resulted in the evaluation of a number of Central American woods for natural resistance to marine (and terrestrial) wood degraders [3,4]. Heartwood extractives from those woods possessing natural marine borer resistance are being evaluated in the field for antiborer activity. It is also important to determine how the active compounds from these resistant woods upset marine borer physiology. Such information could lead to the development of better antiborer agents. By suitable molecular alteration, the natural protectants might be made structurally simpler, more effective, and more economically produced for commercial use. Physiological studies require the development of stable and sensitive techniques, and experiments are best conducted with standardized laboratory cultures of organisms in order to establish the degree of experimental control necessary for quantitative, reproducible work. Such laboratory studies, and the molecular manipulation of a natural product, have been performed elsewhere on the larvae of the marine borer *Lyrodus pedicellatus* [5,6] using obtusaquinone, a natural antiborer extractive from the wood of *Dalbergia retusa*. This paper describes the establishment and maintenance of a closed, recirculating aquarium system for the support of a laboratory culture of the crustacean *Limnoria tripunctata*. This isopod will be used in similar studies involving the extractives from woods specifically resistant to it.

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Fig. 1 — Wooden structures in San Francisco Bay destroyed by marine boring organisms: (a) railroad trestle, (b) ferry slip, and (c) municipal wharf and pier [1]. Photo courtesy of the American Wood-Preservers Assn.



**Fig. 2 — Limnorian-damaged piling showing the characteristic damage pattern produced by these organisms in the intertidal zone [1]. Photo courtesy of the American Wood-Preservers Assn.**

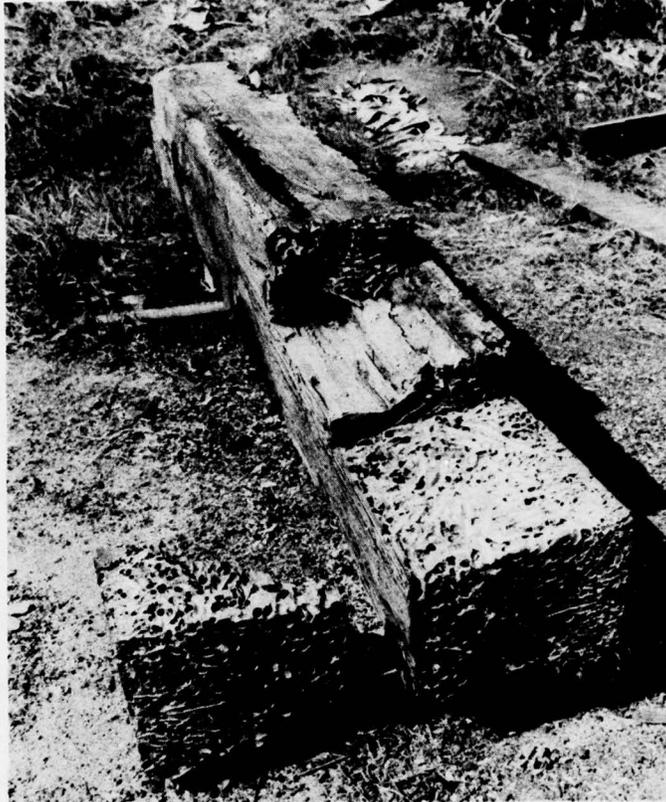


Fig. 3 — Fender timber thoroughly infested by marine borers. The outer surface gives little indication of the extent of internal damage.

## BACKGROUND

### Other Culture Techniques

Laboratory culturing of marine bivalves was first attempted in the last century, but few workers succeeded in rearing them to metamorphosis. In 1927, Wells [7] reared the American oyster *Crassostrea virginica* from artificially fertilized eggs to the settlement and shell-building stage, but his results were not repeatable. As late as the 1950's, culturing depended upon using planktonic larvae instead of obtaining them from fertilized eggs under controlled laboratory conditions. Most recently, Loosanoff and Davis [8] and Walne [9] employed culture techniques which enabled them to culture successfully the larvae of bivalves. However, their laboratories were located at the seashore, and the culture systems utilized running natural seawater. Turner, a leading authority on the laboratory culture of teredinids and limnoriids, also uses water piped from the sea [10].

In open marine culture systems, the toxic organic nitrogenous compounds excreted by the animals are continuously carried off by fresh seawater which passes over the animals once. Laboratories not close to the seashore must use closed, recirculating aquarium systems. These offer the advantages of independence from variations in salinity, temperature, plankton blooms, and the increasing levels of pollution occurring in the near-shore environment from which seawater for culture systems is usually drawn.

Many laboratories and commercial aquaria use recirculating tanks for culture and display purposes. The design parameters for monitoring these tanks have been presented by several investigators [11-16]. Some of the standard techniques have been modified for the present work to accommodate the special problems encountered in our culturing of marine crustacean borers.

### Biology of Limnoriids

Limnoriids, or gribbles as they are commonly called, are small isopods belonging to the class Crustacea. They average about 0.5 mm in diameter  $\times$  3 mm, the male being slightly smaller than the female. The body (Fig. 4) is divided into three main segments which are the head, thorax, and abdomen. The head possesses six sets of appendages. These include the mandibles and first pair of maxillae which are equipped with sharpened, chitinized tips and strong muscles for chewing. The thorax contains seven pairs of walking legs ending in claws which are used to grasp the wood. During the breeding season a brood pouch develops on the females between the second and fifth swimming legs. Fertilized eggs are deposited here and develop into embryos and larvae. The abdomen bears six pairs of legs called pleopods. These pleopods are equipped with gills which are constantly in motion, causing a circulation of water within the burrow. This water is enriched with oxygen which is adsorbed and used by the animals for metabolism.

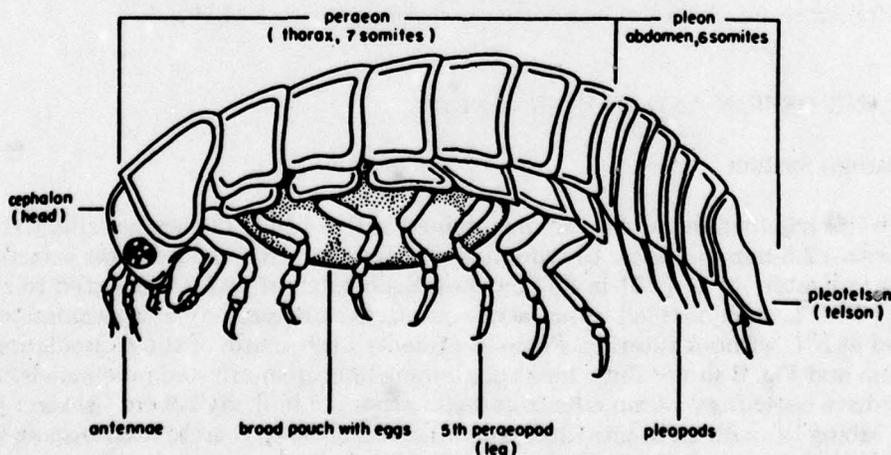


Fig. 4 — Morphological representation of a limnoriid, showing the whole animal, lateral view. (from D.L. Ray, (ed.), *Marine Boring and Fouling Organisms*, with permission of the University of Washington Press, 1959).

Limnoriae are continually active and once a new burrow is begun, the animals are usually enclosed within the wood in 4 to 6 days. Young adults leaving the parental burrow immediately begin boring at right angles to it. The burrows, which are just beneath and parallel to the wood surface, are marked at regular intervals by small breathing holes not large enough for the animals to pass through. These holes serve as passages for oxygen-containing water which is circulated through the burrows by the beating action of the pleopods.

No wood debris accumulates at the closed end of a burrow, and all wood fragments scraped away are ingested and pass through the alimentary canal [17]. Though limnoriae were once thought to burrow into wood solely for protection, subsequent studies have indicated that they are capable of utilizing the wood scrapings as food. The wood is hydrolyzed by a cellulase which is produced by the borers [18] rather than by cellulase-producing microbial symbionts in the gut. In fact, Ray [19] showed that no microorganisms are present in the alimentary tract of these animals. Scanning electron microscope studies by Sleeter, et al. [20] have confirmed these observations. They also showed that there are no microorganisms at the anterior ends of the burrows; the boring activity of the animals is apparently rapid enough to prevent the colonization of the newly-formed wood surface. Limnoriae require nitrogen for amino acid and protein synthesis, and wood is low in this element. Thus, if limnoriae utilize wood as a food, their nitrogen requirement must be satisfied from another source. Marine fungi have been considered because these nitrogen-rich microorganisms are ubiquitous; another suggested source is the pool of dissolved nitrogenous compounds (ammonia, nitrites, nitrates) in the water [21].

Lignicolous marine fungi (or bacteria) may have some relationship to limnoriae other than nutritional. It has been hypothesized that before marine borers can penetrate a wood surface it must be softened by fungal activity, and it has been reported that limnoriae are unable to attack wood in the absence of marine fungi [22]. However, it was demonstrated subsequently that limnoriae are capable of growing in fungi-free wood [23,24]. The consensus is that marine fungi are not necessary for limnorian attack on wood, although a fungal-softened wood surface would certainly increase the rate of attack.

## BASIC TANK DESIGN AND MAINTENANCE

### The Aquarium System

*Limnoria tripunctata* cultures are maintained in five 114-l, all-glass aquaria, tightly covered with 12.5-mm plexiglass to minimize evaporation. Artificial seawater is made up according to Kester, et al. [25] in 45-l batches. Each batch of water is adjusted to a final salinity of 30.0‰ with distilled water, aerated until the pH is steady at approximately 8.2, and stored at 3°C without filtering. Figure 5 presents a schematic of the recirculating culture system and Fig. 6 shows three tanks containing limnorian-infested pine panels. A small magnetic-drive centrifugal pump circulates water from the tank via 1.9-cm (3/4-in.) polyethylene tubing through an in-line filter containing filter fiber, then to a sidearm at the base of the biological filter bed (BFB). Culture water is biologically and mechanically filtered through the BFB before it returns to the tank.

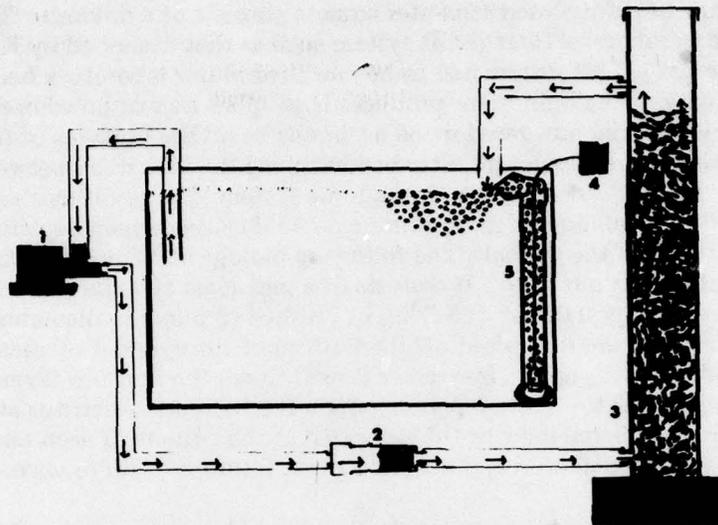


Fig. 5 — Schematic view of our closed-system aquaria for maintaining limnorian cultures. Water from the tank is moved by an all-plastic centrifugal pump (1) through an in-line filter containing charcoal and plastic wool (2) which removes larger particulate matter, to the bottom of the vertical biological filter bed (3) of crushed oyster shells, and then back into the tank. The water is oxygenated and the intratank circulation improved by air from an air pump (4) which is routed through an air-lift tube (5).

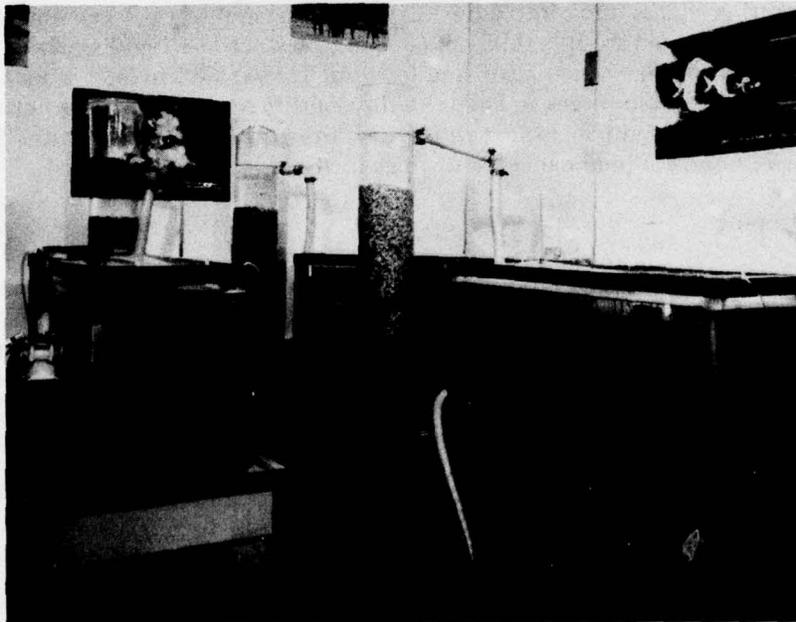


Fig. 6 — Three laboratory culture tanks containing limnorian-infested pine panels suspended by glass hooks from horizontal support rods. The vertical columns are the biological filter beds containing the crushed oyster shells.

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The standard BFB for closed saltwater aquaria consists of a dolomite filtrant used in conjunction with a subgravel filter/airlift system such as that described by King and Spotte [26] and Spotte [27]. This system had to be modified in our laboratory because the constant boring activity of the limnarians produces large quantities of powdered wood in addition to fecal material. The powdered wood accumulates on the bottoms of the tanks and subsequently would cover the entire filter bed, clogging the interstices between the gravel and preventing efficient flow of water through the system. The resulting stagnation of the water and the ensuing buildup of toxic ammonia could cause oxygen depletion, a rise in pH, and eventually, death of the animals. The following biological filtration system was developed for each tank to fit our needs. It consists of a plexiglass cylinder (14-cm I.D.  $\times$  1.5 m) filled with approximately 0.02 m<sup>3</sup> (18.2 kg) of crushed (2.5 mm in diameter) commercial oyster shells. The shells are supported off the bottom of the cylinder by means of a perforated plexiglass plate that permits free water flow through the first few layers of shells. The flow rate ranges from 12 to 60 l/h depending upon the buildup of detritus at the base of the BFB. Three air-pump-operated airlift tubes located at the corners of each tank provide adequate intratank water circulation and keep the water saturated with oxygen.

The salt water in the tanks is kept at the desired salinity by periodic addition of distilled water. Oxygen saturation, pH, and toxic chemical buildup are monitored weekly. Toxic chemicals are further controlled by monthly replacement of 25% (19 l) of old culture water with fresh seawater. The in-line filters are cleaned twice weekly, and the tank bottoms are vacuumed monthly to remove all frass. Also, the BFB is dismantled and cleaned with salt water bimonthly. Temperature is maintained at 24.0°C  $\pm$  1.0°C and light at 400–500 lux is supplied 12 h per day. There are no metals in direct contact with the culture media. Wood panels used for culturing the borers are prevented from floating at the water surface initially, or lying on the bottom because of a subsequent decrease in wood buoyancy. This is accomplished by inserting a glass hook into a hole drilled at one end of each panel which is then anchored to, or suspended from, a 0.95-cm (3/8-in.) plastic rod extending the length of the tank; there are two rods each at the top and bottom. These rods are held in place by a plexiglass framework which also serves to keep the three airlift tubes in the tank corners in a vertical position. The wood panels are riddled with burrows in about 6 months' time as is shown in Fig. 7; a control pine panel is shown at lower right.

### Water Conditioning

A newly established aquarium is conditioned for about 1 month before the culture animals are introduced. During this time the new filter system is exposed to the excretory products, e.g., urea, of hardy marine organisms such as hermit crabs, brittle stars, urchins, or small fish. This conditioning process is accelerated by the addition of some bacterially contaminated oyster shells from an already operating aquarium. This oyster-shell inoculum provides the beginning of a rapidly increasing population of heterotrophic bacteria which become suspended in the water column and attached to the shell surfaces in the BFB. The organic nitrogenous compounds (urea) produced by the "conditioning" animals are rapidly converted by these bacteria into ammonia which reaches a high concentration in the water shortly after the animals are introduced into the system. This mineralization reaction is the first stage of the biological removal of toxic metabolites (Fig. 8).

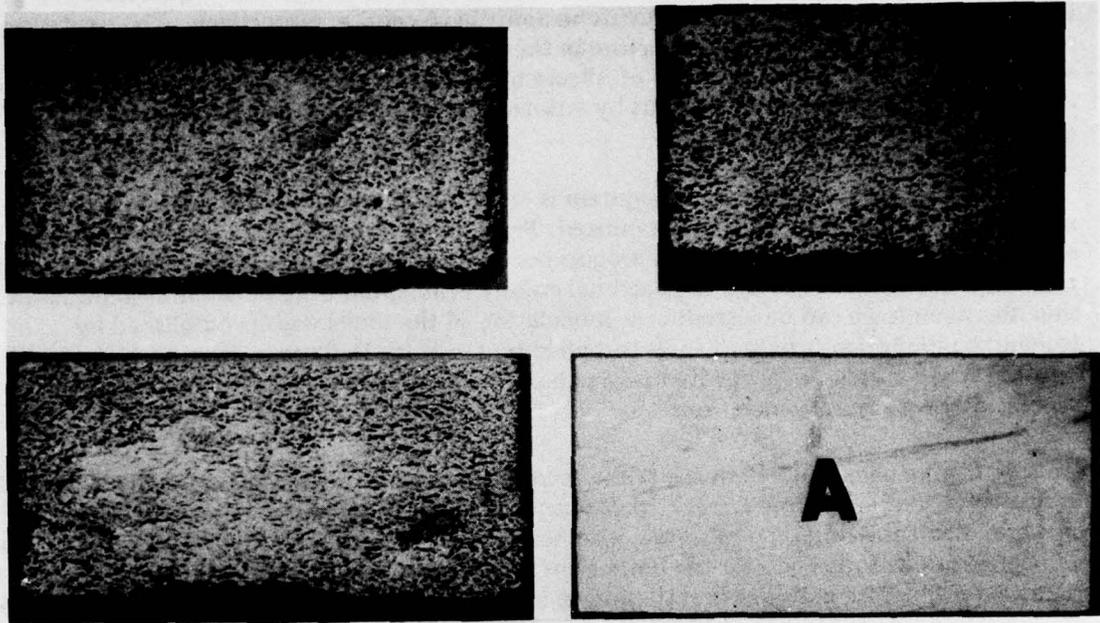


Fig. 7 — Attacked from both sides, these pine panels were reduced from sound wood (A) to spongy masses after 6 months' exposure in our laboratory colony of *Limnoria tripunctata*.

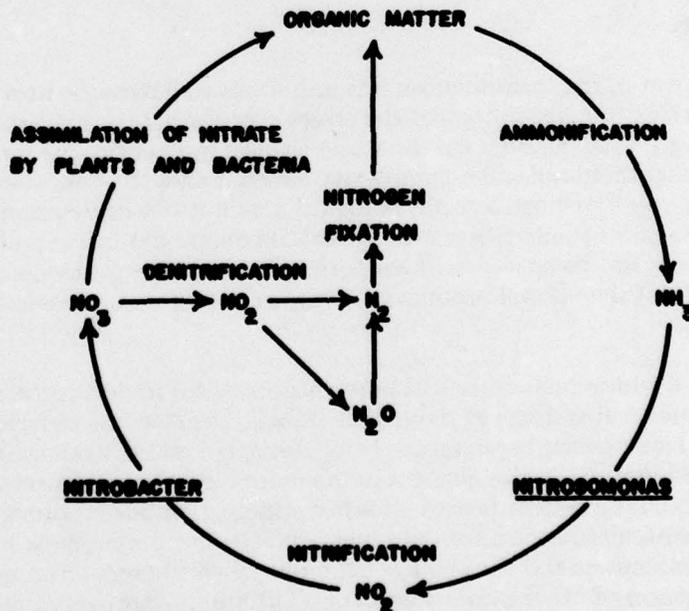


Fig. 8 — Scheme for biological removal of harmful nitrogenous metabolites which accumulate in the water (from S.H. Spotte, *Fish and Invertebrate Culture*, John Wiley and Sons, Inc. 1970).

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The high concentration of ammonia stimulates a rapid increase in the number of the supplied nitrifying autotrophic bacteria *Nitrosomonas* sp. and *Nitrobacter* sp. which perform the oxidation of ammonia to nitrite and nitrite to nitrate, respectively. The oxidation of ammonia to nitrate is the second stage in the biological filtration of the water. The final stage in this process is the reduction of nitrate to nitrous oxide or elemental nitrogen. This denitrification step can be carried out by autotrophic or heterotrophic bacteria and can occur aerobically or anaerobically.

Within 2 weeks to a month the system is conditioned and the concentrations of ammonia, nitrite, and nitrate have stabilized. There is no longer a lag time between successive steps in the nitrogen conversion process because all the nitrogen intermediates are being processed simultaneously, and the bacterial colony is in equilibrium with the system. At this time the limnarians can be introduced. Inoculation of the tanks was accomplished by placing the limnarian-infested panels (supplied by Dr. Ruth D. Turner, Harvard University) into the water among the untreated pine panels. These animals have now been cultured successfully for several generations.

New pine panels placed in the tanks become infested by a euryhaline fungus identified as an *Aspergillus* sp. Within 2 weeks these panels are 70-80% covered with black hyphae and conidia of the fungi. To reiterate, whether these organisms serve a purpose other than as possible source of nitrogen for the limnarians is debatable [28-31]; any beneficial effects of a mechanical nature ensuing from the fungal infection of a wood surface are probably more important to settling teredinid larvae than to young limnarians which become established in the wood by an entirely different mechanism.

## FUTURE WORK

As pointed out in the Introduction, it is important to determine how active natural wood products affect the physiology of the target organisms. Often natural products are present only in very small amounts in the wood tissues; frequently, the problem is compounded further by their molecular complexity which makes the cost of commercial synthesis prohibitive. By knowing their physiological action it can be determined if the biological activity is resident only in a certain chemical constituent in the molecule or if the molecule must be intact to be active. If the former is true, there is the possibility of duplicating the activity of the natural product in a simpler compound, more easily and economically synthesized.

Our in-house limnarian culture will provide animals for physiological studies to determine which biochemical systems of these animals, e.g., the electron transport system or the cellulase (wood-decomposing) system, are being disrupted and to evaluate the effects of synthetically produced molecular changes in the natural products. Concentrated studies will be made on the cellulase system because it is less widely distributed among creatures in the marine environment, and hence a toxicant which is effective only against it will damage fewer nontarget organisms and thus be less offensive, environmentally. A continuing phytochemical examination of other bioresistant wood [3] will be strongly oriented toward those woods specifically resistant to limnarians. Cocobolo, guayacan, and laurel negro are already being examined for their antilimnarian component; lignum vitae, zaragosa, and quirá will be added to the list.

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SUMMARY

The Naval Research Laboratory is studying the nature of the natural resistance of certain tropical woods to wood destroyers, particularly marine borers. Active constituents of resistant woods are being isolated and characterized, and their mode of action on the target organisms determined. Part of this work involves the use of experimental animals. A closed, recirculating, synthetic-seawater aquarium system suitable for establishing and maintaining an in-house culture of the limnorian *Limnoria tripunctata* is described.

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